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Prolyl hydroxylation- and glycosylation-dependent functions of Skp1 in O_2-regulated development of *Dictyostelium*

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ABSTRACT

O_2 regulates multicellular development of the social amoeba *Dictyostelium*, suggesting it may serve as an important cue in its native soil environment. *Dictyostelium* expresses an HIFα-type prolyl 4-hydroxylase (P4H1) whose levels affect the O_2-threshold for culmination implicating it as a direct O_2-sensor, as in animals. But *Dictyostelium* lacks HIFα, a mediator of animal prolyl 4-hydroxylase signaling, and P4H1 can hydroxylate Pro143 of Skp1, a subunit of $E3^{SCF}$ubiquitin-ligases. Skp1 hydroxyproline then becomes the target of five sequential glycosyltransferase reactions that modulate the O_2-signal. Here we show that genetically induced changes in Skp1 levels also affect the O_2-threshold, in opposite direction to that of the modification enzymes suggesting that the latter reduce Skp1 activity. Consistent with this, overexpressed Skp1 is poorly hydroxylated and Skp1 is the only P4H1 substrate detectable in extracts. Effects of Pro143 mutations, and of combinations of Skp1 and enzyme level perturbations, are consistent with pathway modulation of Skp1 activity. However, some effects were not mirrored by changes in modification of the bulk Skp1 pool, implicating a Skp1 subpopulation and possibly additional unknown factors. Altered Skp1 levels also affected other developmental transitions in a modification-dependent fashion. Whereas hydroxylation of animal HIFα results in its polyubiquitination and proteasomal degradation, *Dictyostelium* Skp1 levels were little affected by its modification status. These data indicate that Skp1 and possibly $E3^{SCF}$ubiquitin-ligase activity modulate O_2-dependent culmination and other developmental processes, and at least partially mediate the action of the hydroxylation/glycosylation pathway in O_2-sensing.

Introduction

When starved, cells of the social amoeba *Dictyostelium* aggregate and form a migratory slug, which subsequently culminates into a sessile fruiting body composed of tens of thousands of spores supported above a narrow cellular stalk. In the native environment of the soil, this asexual developmental pathway provides a mechanism for normally subterranean, solitary amoebae to synergistically achieve an aerial disposition from which spores may disperse to distant locales to renew proliferation. The slug-to-fruit switch decision critically depends on O_2-concentration (Sandona et al., 1995) and other environmental factors such as NH_3, light, humidity and warmth, some of which have been shown to signal via protein kinase A (Kirsten et al., 2005). Whereas only 2.5% O_2 is required for proliferation, 10–12% O_2 is required for development of the normal strain Ax3 past the slug stage if cells reside at an air–water interface (West et al., 2007), and 70% is required for terminal differentiation into stalk and spore cells when cells are submerged (West and Erdos, 1988).

The O_2-set point for culmination appears to involve signaling via P4H1, the *Dictyostelium* ortholog of HIFα prolyl 4-hydroxylase (PHD or HPH) (West et al., 2010), a major O_2-sensor of animals (including humans) involved in mid-to-long term responses to hypoxia (Kaelin and Ratcliffe, 2008). Disruption of the *phyA* gene encoding P4H1, or increased P4H1 enzyme activity due to overexpression, causes an increased or decreased O_2-requirement for culmination, respectively. However, *Dictyostelium*, and other protists that possess *phyA*-like genes, lack HIFα, the transcriptional factor subunit that is destabilized by hydroxylation of Pro-residues in its two O_2-dependent degradation domains. A known substrate for *Dictyostelium* P4H1 is Skp1 (van der Wel et al., 2005), a subunit of the SCF-class of E3 Ub-ligases. $E3^{SCF}$Ub-ligases regulate the cell cycle, nutrient sensing, physiology and development in many organisms (Willems et al., 2004), including the latter in *Dictyostelium* (Ennis et al., 2000; Nelson et al., 2000; Mohanty et al., 2001; Tekinay et al., 2003). Dd-Skp1 is modified at Pro143, which is replaced by Glu in chordate Skp1s. Interestingly, $E3^{SCF}$Ub-ligases are evolutionarily related to the $E3^{VHL}$Ub-ligase which mediates O_2-

Abbreviations: GlcNAc, N-acetyl-D-glucosamine; HIF, hypoxia inducible factor; Hyp, 4-hydroxyproline; PHD, prolyl hydroxylase domain protein; RFP, red fluorescent protein; rP4H1, recombinant His_6P4H1 isolated from *E. coli*; rGnt1, recombinant His_6Gnt1 isolated from *E. coli*; Ub, ubiquitin.

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dependent degradation of animal HIFα in normoxia (Kaelin and Ratcliffe, 2008), suggesting a potentially related signaling mechanism associated with protein stability. Therefore Skp1 is a candidate for mediating the O_2-signaling role of P4H1 in *Dictyostelium*.

Hydroxylated Skp1 is subject to successive further modification by three gene products, resulting in the assembly of a pentasaccharide on the fully processed protein (see Fig. 1A). Disruption of the dual function glycosyltransferase gene *pgtA*, which results in accumulation of Skp1 whose Hyp is modified by the single sugar GlcNAc, leads to a near wild-type O_2-dependence that originally suggested that peripheral glycosylation is not relevant to O_2-dependent signaling (West et al., 2007). However, a recent study showed that disruption of *agtA* (Ercan et al., 2006), required for addition of the final two sugars, results in dependence on high O_2 approaching that of *phyA*$^-$ cells, revealing a modulatory role for glycosylation (Wang et al., 2009). Since Skp1 is the only substrate detected for the PgtA and AgtA glycosyltransferases in biochemical screening studies, Skp1 is implicated as the functional target of P4H1 in O_2-signaling as well. However, since animal PHDs appear to have multiple substrates in O_2-signaling (Kaelin and Ratcliffe, 2008), and it is challenging to identify PHD targets, further evidence is required to confirm the hypothesized involvement of Skp1 in P4H1 signaling in *Dictyostelium*.

Wild-type strains of *D. discoideum* harbor two Skp1 genes, Skp1A and Skp1B, whose amino acid sequences are identical except for a difference at codon 39 (Ser/Ala) in the N-terminal region (West et al., 1997). The axenic strain Ax2 possesses the wild-type complement, whereas strain Ax3, used in all studies to date, has two Skp1B genes owing to a 100 kb duplication in chromosome 2. The two genes share the same expression pattern in the life cycle based on RT-PCR and protein studies (Sassi et al., 2001; West et al., 1997), and are conserved in the genomes of four other social amoebae (unpublished data). Using reverse genetic approaches, we find that decreasing or increasing Skp1 levels modulates the O_2-dependence of culmination over the same range affected by changes in P4H1 levels, except in an opposite direction suggesting that hydroxylation opposes Skp1 activity. Other developmental steps are also affected by changes in Skp1 levels. Enzymatic assays and the phenotypes of Pro143 point mutations, and combined mutations affecting both Skp1 and modification pathway enzymes, are consistent with a model that Skp1 mediates pathway activity in O_2-regulation. Analyses of the modification status of the bulk pool of Skp1 suggest, however, that the effects are mediated by a subpopulation of Skp1, and leave open the possibility that other targets of the modification pathway also contribute to signaling.

Experimental procedures

Cell growth and development

Strains (Supplementary Table 1) were grown axenically in HL-5 medium on orbital shakers. For development, vegetative cells ($\leq 5\times10^6$ cells/ml) were centrifuged at 1000 $g\times1$ min, resuspended in ice-cold PDF buffer, centrifuged again, and resuspended in PDF (West et al., 2007). 0.4 ml cells (10^8/ml) were spread on 47 mm-diameter Millipore filters in 60×15 mm Petri dishes and incubated in sealed plastic boxes, under overhead room fluorescent lighting at 22 °C, for up to 46 h in the presence of the indicated concentration of flowing O_2 with the balance made up with N_2. Development was evaluated morphologically and by counting spores in a hemacytometer.

Stationary stage cells were collected at $2–3\times10^7$/ml. For aggregation stage cells, washed vegetative cells were resuspended at 2×10^7/ml in 2 ml of Agg buffer (0.01 M $NaPO_4$, 0.01 M KCl, 0.005 M $MgCl_2$, pH 6.0), and shaken in a flask for 8 h. Slug stage cells were scraped from filters 2–3 h after their initial appearance.

Shaking cells were incubated with 400 μg/ml cycloheximide (unless otherwise stated) from Sigma Chemical Co., diluted from a 50 mg/ml stock solution in DMSO, for the indicated time. Control cultures were incubated in 0.8% DMSO. For metabolic labeling, 5 μCi of ^{35}S-Met (carrier-free, Amersham) was added 15 min after introduction of cycloheximide and incubated for 2 h. Incorporation into protein was measured by TCA precipitation as before (Sassi et al., 2001).

Cell extracts and protein analyses

For standard Western blot analysis, cells were collected by centrifugation (1000 $g\times1$ min), resuspended in ice-cold 50 mM Tris–HCl, pH 8.0, centrifuged at 5000 $g\times15$ s, and the pellet frozen at −80 °C. For protein determination, pellets were resuspended in ice-cold 50 mM Tris–HCl, pH 8.0, 10 mM EDTA, 0.1% NP40, 0.5 mM

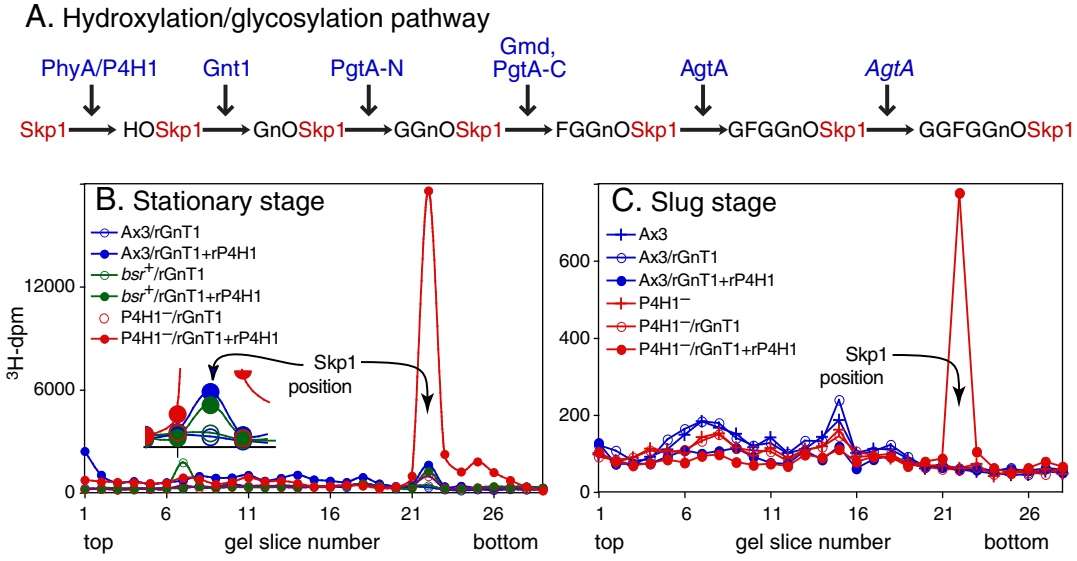

Fig. 1. Specificity of P4H1 and Gnt1. (A) Schematic of the hydroxylation/glycosylation pathway, using Skp1 as a target example (West et al., 2010; *AgtA*, unpublished data). (B) Soluble extracts (S100) of *phyA*(P4H1)$^-$ stationary stage cells were desalted, incubated with rP4H1, rGnT1, and enzyme substrates and cofactors including UDP-[^{3}H]GlcNAc. Controls included parental Ax3 cells (P4H1$^+$), *bsr*$^+$ cells from a transfection with an empty plasmid, and reactions lacking P4H1. After 2 h, a reaction aliquot was subjected to SDS-PAGE and sliced into bands that were counted for radioactivity. Migration position of Skp1 is indicated. Inset shows low-level P4H1/Gnt1-dependent incorporation at the position of normal cell Skp1. (C) Similar analysis of slug cells.